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13. ABSTRACT (Maximum 200 Words) The PTEN tumor suppressor gene is among the most commonly mutated genes in prostate cancer. PTEN antagonizes the PI3K/AKT pathway by dephosphorylating the 3' position of phosphoinositides. We had previously found that phosphorylation of the PTEN Tail regulates its membrane recruitment by regulating its interaction with a protein complex. The main aim of this proposal is to identify the components of this protein membrane complex and test their functional role in the PI3K pathway (apoptosis or cell cycle) in prostate cancer cell lines. Importantly, in the first year of the project we have found two alleles of PTEN that either enhance or decrease membrane recruitment. We have also generated expression plasmids containing these mutations with the Flag-His tag system to be used in the immunoaffinity purification experiments. Notably, we have determined the optimal conditions to use in the PTEN purification protein complex. We have found that the use of a specific crosslinker prior to the lysis of the cells is essential for the stabilization of the PTEN protein complex. Finally, we have performed a large-scale purification using these conditions and found several specific bands interacting with PTEN that are currently being sequenced.		
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INTRODUCTION

To successfully treat patients with advance stage prostate cancer we need to develop effective therapeutic agents. To be able to do so, we need to understand the genetic and molecular changes that occur in the process of prostate cancer development. Phosphatase and Tensin homologue (PTEN) was cloned and mapped to chromosome 10q23, a region that is frequently altered in prostate cancer (Parsons and Simpson 2003). Since the cloning, an increasing number of reports have demonstrate that PTEN plays a very important role in the development of prostate cancer (Visakorpi 2003). PTEN dephosphorylates the 3' position of phosphoinositides and by doing so antagonizes the PI3K pathway (Vazquez and Sellers 2000). Although it is clear that the substrate of PTEN is at the plasma membrane how PTEN translocates to the plasma membrane and how it is activated is currently unknown. We previously demonstrated that PTEN is phosphorylated at the C-terminal Tail and that this phosphorylation regulates its conformation and recruitment to the plasma membrane (Vazquez, Ramaswamy et al. 2000) (Vazquez, Grossman et al. 2001). The central aim of this proposal is to elucidate the components responsible for PTEN recruitment to the plasma membrane. We will test the hypothesis that proteins that regulate PTEN will influence its tumor suppressor function. In particular, 22% of prostate tumors have lost PTEN protein, however PTEN may be deregulated in those tumors that retain intact PTEN protein because of genetic changes in the interacting proteins.

BODY

Identification of PTEN domains important for plasma membrane binding

We have taken advantage of the fact that PTEN is highly conserved in *Dictyostelium discoideum* (*D. discoideum*). This is a more simple system for biochemical analysis and we can grow large quantities of cells in a few days instead of weeks. A major breakthrough occurred when we expressed Human PTEN in *D. discoideum* *pten* - cells and found that it completely restored PTEN's function to the cells. This implies that the specific protein-protein interactions important for PTEN tumor suppressor activity are taking place in these cells. Importantly, we can express and purify large amounts of biological y active PTEN (and interacting proteins) in a short time period. Thus, we can now use *D. discoideum* cells to perform the initial protein complex purification and then apply the knowledge and techniques to prostate cancer cell lines. This will greatly increase the speed of the project.

The identification of the domains that are important for PTEN binding to the plasma membrane is very important for this project as they are more likely to be interacting domains with other proteins. As stated in the grant proposal we have previously found that phosphorylation of PTEN at serine-380, threonine-382 and threonine-383, and serine 385 (C-terminal phosphorylation sites) inhibit PTEN function by modulating its conformation and localization (Vazquez, Grossman et al. 2001). A recent report has shown that PTEN;C124S (phosphatase inactive mutant) in combination with a mutation of the C-terminal Tail phosphorylation sites, that we have previously identified (what we called PTEN;A4), greatly enhances PTEN binding to the plasma membrane (Das, Dixon et al. 2003).

In order to investigate PTEN membrane localization we have used site-directed mutagenesis, polymerase-chain-reaction (PCR) and restriction cloning to clone the PTEN cDNA tagged with GFP at the C-terminus into *D. discoideum* expression vectors (PTEN;WT-GFP, PTEN;C124S-GFP, PTEN;C124S;A4-GFP, PTEN;Δ10-GFP, PTEN;C124S;Δ10-GFP, PTEN;C124S;A4;Δ10-GFP). We have found that PTEN;C124S;A4 localization is greatly increased at the plasma membrane but PTEN;G129R;A4 is not. These results strongly indicate that is not the inactivation of PTEN phosphatase activity enhancing its membrane localization but rather the phosphatase pocket itself that contributes to the membrane binding. Importantly, we have found that the N-terminal PIP2 domain is critically required for PTEN membrane binding, as deletion of the first 10 amino-acids in the context of the C124S;A4 (PTEN;C124S;A4;Δ10) mutation prevents PTEN from binding to the membrane.

Also, although beyond the proposal of this grant, we have collaborated with Toshio Yanagida at the University of Osaka and using single-molecule microscopy we have seen PTEN;WT binding to the membrane that is nevertheless enhanced by mutation of the Tail phosphorylation sites.

In conclusion for this part of the project, we have made very important discoveries that are key for the outcome of the project. Importantly, we have successfully identified PTEN;C124S;A4 as the allele that is more highly localized at the plasma membrane. Furthermore, we have determined that the N-terminal PIP2 domain is necessary for membrane binding. These

results suggest that PTEN;C124S;A4 is the best allele to use for the purification and identification of the PTEN protein complex, as it is localized at the plasma membrane where PTEN is enzymatically active and functioning as a tumor suppressor. Moreover, PTEN;C124S;A4;Δ10 can be used as negative control since this allele does not bind to the membrane.

Generation of Flag-His expression constructs

The main purpose of the task 1 was to generate expression plasmids for PTEN purification and perform preliminary experiments to test the conditions of the PTEN protein complex purification.

The two important new discoveries described in the previous section forced us to change the initial experimental strategy of using PTEN tagged at the N-terminus with Flag-HA (Flag-HA-PTEN) to a new approach using PTEN tagged at the C-terminus (PTEN-Flag-His).

1. In the grant we proposed the use of a tandem affinity purification with the Flag-HA system to purify the PTEN protein complex. This PTEN tagged protein allows for double immunoaffinity purification using anti-Flag and anti-HA antibodies followed by their respective peptide elution. Notably, several preliminary small-scale experiments were done with this system and although anti-Flag immunoaffinity beads gave consistently good yield immunoprecipitation, the anti-HA beads gave inconsistent results. For this purpose, we have decided to change to PTEN tagged with Flag-His and used it for tandem affinity purification.

2. Importantly, we have also discovered that the N-terminal PIP2 binding domain is required for PTEN binding to the membrane. Therefore, the addition of the tag at the C-terminus, rather than at the N-terminus, is more likely not to interfere with the functional interactions.

We have generated PTEN-Flag-His plasmids as well as several important mutants (PTEN;A4, PTEN;C124S;A4, PTEN;C124S;A4;Δ10) using site-directed mutagenesis and polymerase-chain-reaction (PCR) amplification. These alleles will be used to identify the specificity of the interacting proteins.

Preliminary purification to determine the optimal conditions

Small-scale experiments were performed to determine the optimal conditions of the purification

We lysed 2.10^7 cells expressing PTEN-Flag-His that was purified using Flag antibody linked to agarose beads. In figure 1, a typical experiment result is shown. The amount of Flag beads required for the purification is approximately $1\mu\text{l}$ of Flag beads / 10^7 cells (Figure 1 and data not shown) and that concentration of Flag peptide required for the elution is $100\mu\text{g}/\text{ml}$ of flag peptide. As shown in figure 1, we can further elute PTEN with a low pH and could demonstrate that most, if not all, PTEN can be eluted with this concentration of peptide. Moreover, after several experiments, we have determined that for the first purification step the

amount of N^{+2} resin required to bind PTEN is 10 μ l of Niquel resin/ 10^7 cells. The optimal concentration of Imidazole required for elution is 150mM.

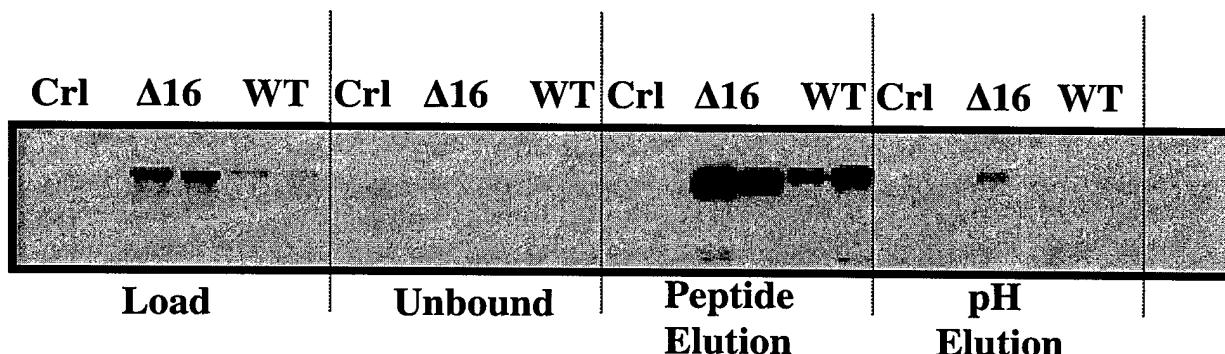


Figure 1. 2.10^7 *D.discoideum* cells wild type (Ctr) or expressing PTEN;WT or PTEN;Δ16 cells were lysed. After clearing 2 μ l of Flag-beads were added to the lysate and incubated 3 for h at 4°C. After extensive washings, bound PTEN was eluted with Flag peptide at 100 μ g/ml. Beads were then further eluted with 100mM Tris-Glycine at pH 3.0. An aliquot of each purification step was runned in an SDS-PAGE gel and western blotted with an anti-Flag antibody.

Medium-scale (2.10^9 cells) experiments were performed to determine the presence of PTEN interacting bands

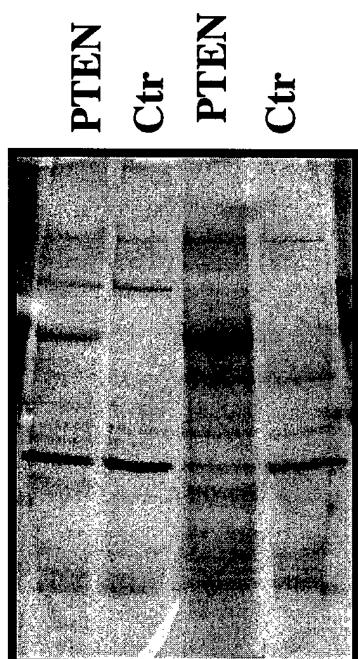


Figure 2. Anti-Flag purification from control cells (Ctr) or cells expressing PTEN (PTEN)

In a typical experiment we lysed 2.10^9 *D. discoideum* cells with a nylon membrane. After a brief centrifugation to remove non-lysed cells and nuclei, we applied a high-speed centrifugation and separated a pellet containing membranes and supernatant. The pellet was solubilized with a buffer containing 1% NP40 and Flag-beads were added and incubated for 3h at 4°C. After extensive washings, proteins were eluted with a flag peptide and separated on SDS-PAGE gel. Gel was then stained with silver to identify the associated proteins (Figure 2). After several experiments, we concluded that although we could detect several interacting proteins we could not reproducibly detect the same bands suggesting that the interactions were very transient or disrupted with the solubilization conditions.

Use of crosslinkers to stabilize interactions

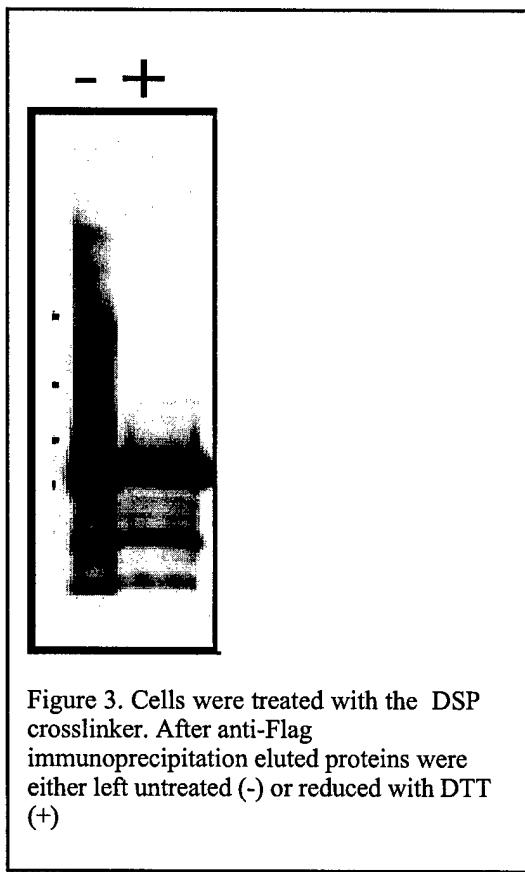


Figure 3. Cells were treated with the DSP crosslinker. After anti-Flag immunoprecipitation eluted proteins were either left untreated (-) or reduced with DTT (+)

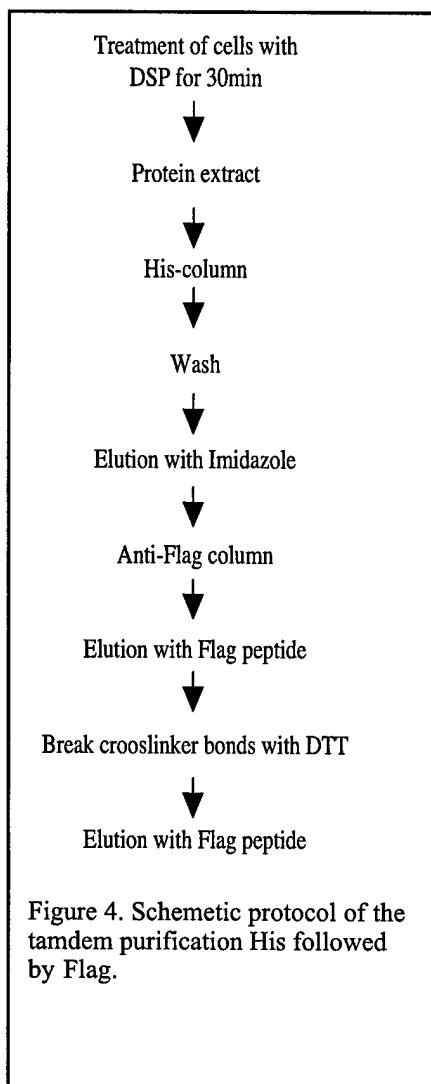
The most difficult task in purifying interacting proteins from signal transduction complexes associated with cellular membranes is that the use of detergents will often disrupt the interactions. The use of detergents is however necessary to solubilize proteins localized at the plasma membrane. Additionally, some protein-protein interactions are very weak and hard to maintain during the purification. A variety of crosslinking reagents available commercially can circumvent this problem.

Specifically for the project of this grant we have tested the Dithiobis(succinimidylpropionate (DSP) crosslinker. This crosslinker has some key features. It is membrane permeable and thus can be used in intact cells. It can also be cleaved with dithiothreitol (DTT) and the reaction can be quenched with Tris buffer.

We then tested whether the DSP crosslinker would help to stabilize the interactions of the PTEN complex. Cells were treated with 2mM DSP for 30min prior to purification. The eluted proteins were

then either left untreated or treated with DTT to disrupt the interactions. Samples were then separated with SDS-PAGE and western blotted with anti-flag antibody. As seen in figure 3, we could detect very specific higher molecular weight bands that disappear after treatment with DTT.

Large-scale purification of PTEN;C124S-Flag-His



A large-scale experiment was performed with *D. discoideum* cells. A schematic protocol is shown in figure 4. Briefly, we started with 2.10^{10} cells. Cells were treated with DSP for 30 min and lysed with a buffer containing 1% SDS to solubilize membrane proteins. Extract was then diluted 10 times with a buffer containing 2% NP40 and cleared by centrifugation. Nickel beads were added to the extract and incubated overnight at 4°C. After extensive washings, proteins were eluted with 150 mM Imidazole. Eluted solution was diluted 50 times and anti-Flag beads were added. After incubation for 3h at 4°C, bound proteins were washed extensively and specifically eluted with Flag-peptide. Bound proteins were separated by SDS-PAGE gel and stained with colloidal coomassie. A band corresponding to the size of PTEN as well as several specific bands could be detected. There were no proteins detected by colloidal coomassie in the control lane indicating that all the bands are specific. All those bands were sliced from the gel and are currently being identified by the mass spectrometry facility.

We will apply these approaches to prostate cancer cell lines in two ways. First, we have developed a very efficient and reliable protocol for the purification of PTEN complexes and we will repeat the experiments with prostate cancer cell lines. Second, as we learn the identity of the interacting protein from the experiments described above, we will determine whether the homologs are expressed in prostate cancer lines and characterize if their can influence PTEN tumor suppressor function.

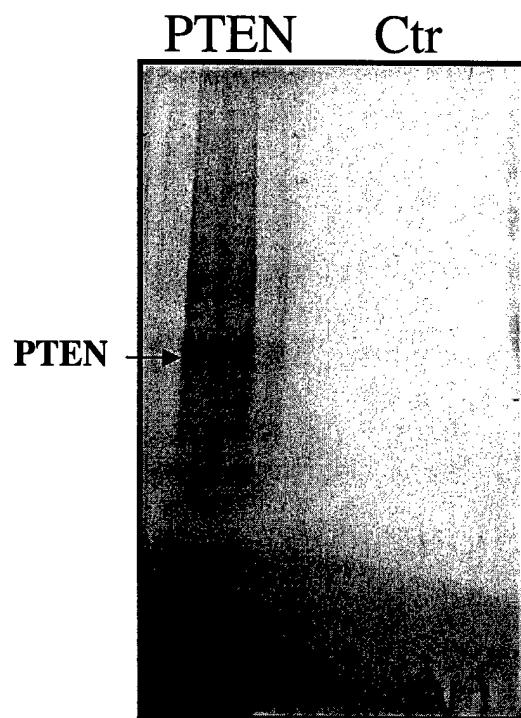


Figure 5. Tandem affinity purification of PTEN

Arrow indicates PTEN protein. Note that several specific bands are detected in along with PTEN and that no bands are detected in the control lane.

KEY RESEARCH ACCOMPLISHMENTS

1. Finding that Human PTEN can complement the phenotype of pten- *D. discoideum* cells. These results clearly indicate that Human PTEN must be interacting with the appropriate partners since it is completely functional.
2. Identification of Flag-His tagged PTEN at the C-terminus (PTEN-Flag-His) as a better experimental approach than Flag-HA at the N-terminus to purify PTEN.
3. Generation of PTEN-Flag-His, PTEN;C124S-Flag-His, PTEN;C124S;A4-Flag-His, PTEN;C124S;A4;Δ10-Flag-His, expression plasmids
4. Development of a protocol to purify PTEN-Flag-His with Anti-flag and Ni⁺² beads.
5. Very important to this project, the discovery that the use of a crosslinker is key to stabilize the interactions and should increase the consistency as well as the amount of interacting proteins.
6. Identification of PTEN interacting proteins in *D. discoideum* cells with the use of the crosslinker. The bands are currently being identified by mass spectrometry analysis.

REPORTABLE OUTCOMES

#2011Abstract to American Association for Cancer Research, 94th Annual Meeting

July 11-14, 2003

Andrew C. Hsieh, **Francisca Vazquez**, Ronghai Bo, Anastasia Khvorova, Stephen Scaringe and William R. Sellers.

Systematic Rnai-mediated inactivation of the components of the PI3K pathway.

Included as Appendix.

CONCLUSIONS

The main aim of this grant is the identification and characterization of proteins that interact and are involved in the recruitment of PTEN to the plasma membrane. These PTEN "interactors" will impact in PTEN function as tumor suppressor and thus help us to further determine the molecular mechanisms of prostate cancer development. During the first year of the project we have discovered that the Flag-His tag is a very good option for purification experiments. We have also discovered that the N-terminal domain of PTEN is required for its binding to the membrane. These two important discoveries made us change from Flag-HA-PTEN to PTEN-Flag-His as a better option for the purification. We have done some small-scale purifications and determine the optimal ratio cells/resin and the optimal concentration of peptide and imidazole for elution of the protein from these resins. We have found that the use of crosslinker prior to the lysis of the cells helps the stabilization of the complex. Using this reagent we have performed a large-scale purification and found several potential interacting proteins that are in the process of being sequenced in the mass spectrometry facility. We will test if these proteins can interfere with PTEN tumor suppressor activity.

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#2011 Systematic RNAi-mediated inactivation of the components of the PI3K pathway. Andrew C. Hsieh, Francisca Vazquez, Ronghai Bo, Anastasia Khvorova, Stephen Scaringe, and William R. Sellers. *Dana Farber Cancer Institute, Boston, MA and Dharmacon Research, Inc., Lafayette, CO.*

The Phosphatidylinositol 3-Kinase (PI3K) signaling pathway is strongly implicated in tumor formation and progression. Specifically, increased serum levels of IGF-1 increase risks for cancer development, Akt and PI3K are amplified in several malignancies, and loss-of-function PTEN alterations are among the most common tumor suppressor mutations in human cancer. These findings demonstrate the critical balance required for pathway homeostasis and that tumor progression is tightly linked to specific pathway changes. Recessive genetic studies in both *C. elegans* and *D. melanogaster* have uncovered two key downstream targets of Akt linked to the process of transformation, including *daf-16* the *C. elegans* homologue of FKHR, FKHR-L1 and AFX, and the *D. melanogaster* homologues of TOR and p70S6K. To date, similar, comprehensive recessive genetic studies of this pathway have not been possible in mammalian cells. The development of efficient RNAi mediated gene knock-down raises the possibility that such approaches may now be feasible. In order to develop methodologies and reagents sufficiently robust for systematic pathway inactivation, 154 siRNA duplexes were designed to 31 putative members of the PI3K pathway. Multiple high-throughput assays capable of scoring multiple substrate phosphorylation events were designed and implemented in a 96-well plate format. Efficient knock-down of each gene was achieved and verified at the protein level. In addition, where suitable functional amplification primers were developed, knock-down of the mRNA was also verified by Q-PCR. In addition, novel and known functional inter-relationships were identified through recessive inactivation. These data will be discussed and presented in detail.